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13. ABSTRACT (Maximum 200 Words)

This project examines the interaction of MUC1, a breast tumor antigen, with APC, a potent tumor suppressor. We have focused on Task 4 in the approved Statement of Work: "To clarify the functional significance of the interaction in relation to β-catenin and ErbB signaling". To this end, we have determined that APC most likely interacts preferentially with wildtype MUC1 as compared to a MUC1 mutant lacking tyrosines in the cytoplasmic tail. We have created several constructs to overcome difficulties associated with poor APC antibodies: His_6- and myc-tagged, as well as constructs for tandem affinity purification (TAP) and fluorescence resonance energy transfer (FRET). We have expressed APC in breast cell lines under a zinc-inducible promoter, but have determined that these cells are too inconsistent to use for immunoprecipitations. In another breast cancer cell lines, MDA-MB-468, we have successfully knocked down MUC1 expression using siRNA technology; interestingly, the reduced level of MUC1 correlated with a decrease in active β-catenin in these cells. Our upcoming studies will make use of TAP, FRET, and siRNA as complementary assays in examining the role of the MUC1-APC interaction in human breast cancer.

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Introduction

MUC1 is a tumor antigen that is overexpressed on a majority of cancers, including over 90% of breast tumors and metastases. It is a large, transmembrane mucin that is heavily glycosylated and expressed as a heterodimer on the apical surface of secretory epithelial layers. The larger portion of the heterodimer consists of the majority of the extracellular domain and ranges from 200 kDa to 1 MDa in size; the smaller portion contains a short extracellular stem, the transmembrane domain, and the 72-amino acid cytoplasmic tail (collectively referred to as MUC1-CT).[1] MUC1 has recently been characterized as an oncogene and has been proposed to possess a number of functions, such as protecting the epithelial layers where it is expressed, modulating cellular adhesion through interactions with sugar-binding proteins and adhesion molecules such as ICAM-1,[2] and participating in cell signaling. This last is accomplished through association with a number of signaling molecules that have been implicated in oncogenesis/tumor suppression, including the members of the epidermal growth factor receptor (EGFR or ErbB) family,[3] glycogen synthase kinase 3β (GSK3β), and β-catenin.[4] These and other proteins capable of binding to MUC1 frequently recognize tyrosine or serine/threonine phosphorylation sites (Figure 1) in the MUC1-CT, indicating that kinase activity may be an important mechanism for controlling the interaction of MUC1 with its binding partners. Association of MUC1 with β-catenin, for example, is thought to be negatively regulated by GSK3β phosphorylation of the MUC1-CT, while tyrosine phosphorylation by EGFR or c-Src stimulates MUC1-β-catenin binding.[5] Interestingly, these interactions likely have physiological significance for breast cancer and metastasis, as Muc1 (note that the mouse protein is designated Muc1 and the human MUC1), β-catenin, and the erbB family interact preferentially in mouse mammary tumors as compared to normal glands.[3, 6, 7] A similar pattern exists in human breast cancer, where these proteins interact in primary tumors and much more strongly in lymph node metastases, while showing little or no interaction in adjacent normal breast tissues.

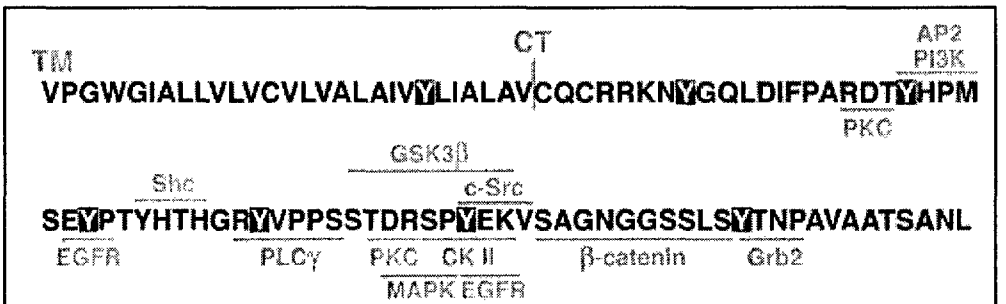

Figure 1: The sequence of the MUC1 transmembrane domaing (TM) and cytoplasmic tail (CT), with protein-protein interaction motifs and tyrosine residues highlighted.

During studies of the interaction of the ErbB family with MUC1 and β-catenin, we observed that MUC1 immunoprecipitations brought down a tyrosine phosphorylated protein of approximately 300 kDa. The fact that MUC1 is known to bind to two members of the Wnt signaling pathway, GSK3β and β-catenin, led us to investigate whether this 300 kDa protein might be another protein in that pathway, APC. APC (adenomatous polyposis coli) is a tumor suppressor that was originally found through studies of hereditary colon cancer.[8] Its best-known function is the regulation of β-catenin levels and localization through association with a multi-protein complex including GSK3β and axin. This complex binds to cytoplasmic β-catenin, resulting in its phosphorylation, ubiquitination, and eventual degradation by the proteasome. The role of APC in this complex is primarily that of a scaffold, although some reports have shown that APC can also bind to nuclear β-catenin and shuttle it to the cytoplasm for degradation. Regulation of β-catenin is extremely important in both the colonic and mammary epithelia, as the protein is heavily involved in cellular adhesion and can act as a transcription factor for targets such as c-Myc and cyclin D1. Excessive β-catenin can be oncogenic.

This project was designed to study the interaction of MUC1 with APC. The primary focus is to look at this association in the context of larger signaling networks, in particular the Wnt/β-catenin and ErbB pathways. β-catenin is a promising target to analyze, since both MUC1 and APC are known to affect its signaling capacity in breast cancer, while preliminary evidence indicated that epidermal growth factor (EGF) treatment may increase the level of MUC1-APC association in lactating mammary glands and breast cancer cells. In addition, our work has shown that interaction of MUC1 and APC follows a similar

pattern to that described above for the binding of MUC1, β-catenin, and the ErbB family: little or no interaction in adjacent normal breast tissue, visible interaction in primary tumors, and much stronger interaction in lymph node metastases. Interestingly, we have only detected MUC1-APC interaction in metastatic human breast tumors and not in node-negative tumors, indicating that this association may serve a functional role in promoting breast tumor metastasis. Given that high levels of either MUC1 or β-catenin are negative prognostic indicators in breast cancer,[1, 9] and that MUC1-β-catenin association is stimulated by known oncogenic signals such as EGFR- or Src-mediated phosphorylation, we hypothesize that MUC1 may play a protective role regarding β-catenin activity in tumors. MUC1 association with APC may therefore represent one aspect of this protective role; for example, MUC1 could sequester APC and/or β-catenin away from the rest of the destruction complex, or, at high levels, it could sequester APC from β-catenin by binding to both molecules individually. As will be presented below, our studies of the MUC1-APC association have also revealed indications that MUC1 may have a more direct role in regulating the activity of β-catenin.

successful, stable transfection of APC into a cancer cell line. Our thought was that even a marginal increase in the amount of APC would greatly aid our studies, as commercial antibodies to this protein are generally of poor quality (described below). The ZR-75-1 cells were chosen because analysis of a panel of breast cancer cell lines (Figure 4) showed co-immunoprecipitation between MUC1 and APC in those cells. This co-immunoprecipitation was stimulated by exogenous EGF treatment.[11] The MDA-MB-468 line was also chosen because of its vigorous response to EGF signaling (these cells express large amounts of EGFR);[12] we hoped to use these cells to determine whether modulating the EGF signaling pathway could induce an interaction between MUC1 and APC. The APC and lacZ constructs were driven in these cells by the cytomegalovirus promoter (pcDNA3.1 vector, Invitrogen) for high-level expression. However, our analysis of the stable transfectants showed negligible increase in the amount of APC protein by western blot (not shown). For this reason, we decided to try a different approach to stable transfection.

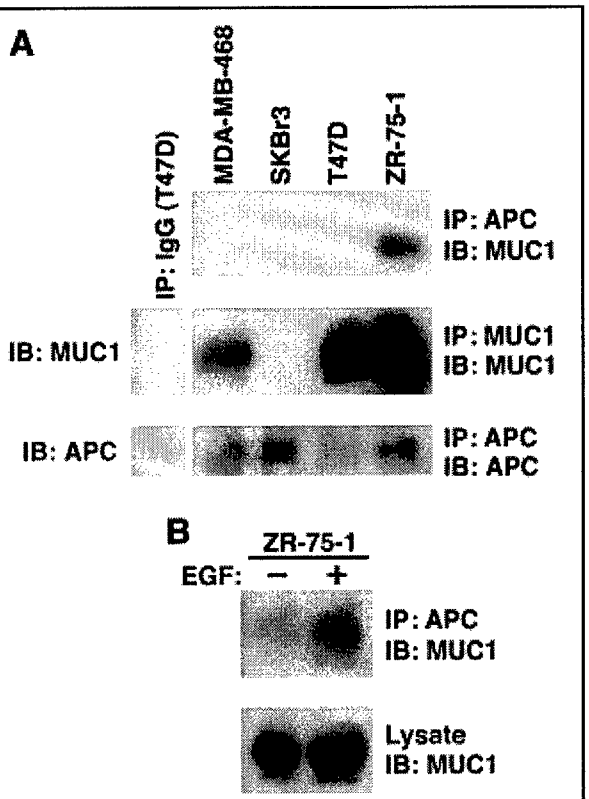

Figure 4: **A.** Co-immunoprecipitation of MUC1 and APC from ZR-75-1 cells. **B.** Epidermal growth factor stimulation of ZR-75-1 cells.

We obtained constructs expressing APC or lacZ under a ZnCl-inducible metallothionein promoter, the pSAR-MT-APC and pSAR-MT-βgal plasmids[13] (kind gifts of B. Vogelstein at Johns Hopkins University). These were transfected into the same breast cancer cell lines and analyzed, initially as a polyclonal population while single-cell clones were developed. As the pSAR-MT constructs do not themselves contain an antibiotic resistance gene for selection, cells were co-transfected with empty pcDNA3.1 vector for neomycin resistance. ZR-75-1 cells were selected for transgene uptake with 1.5 mg/mL G418, while MDA-MB-468 cells were selected with 0.5 mg/mL G418. Even as a polyclonal population, the ZR-75-1 cells showed readily inducible expression of APC with increasing exposure to 100 μM ZnCl (Figure 5). As the MDA-MB-468 cells showed neither a clear increase in APC expression nor a novel co-immunoprecipitation between MUC1 and APC (not shown), we continued our analyses on the ZR-75-1 line with the lacZ-inducible cells as a control. Initial co-immunoprecipitation studies in the ZnCl-inducible ZR-75-1 cells were promising in that we saw an increase in the amount of MUC1-APC association in the APC-expressing line as compared to the lacZ control. However, as we tried to optimize the system (e.g., concentration of ZnCl, length of treatment prior to harvest, etc.), we were unable to see a consistent pattern in the behavior of the cells. Repeated experiments would show great variation in the amount of MUC1-APC, MUC1-β-catenin, and APC-β-catenin co-immunoprecipitation for a given condition relative to controls. We used MgCl treatment as an additional control for the Zn^{2+} induction, but still could not establish a consistent response from the cells. Finally, over time the ZR-75-1 cells no longer showed increased APC expression with ZnCl induction. This problem was not resolved by thawing fresh cells and is likely due to gradual loss of the transfected APC construct. Cells were maintained in selection throughout the length of these experiments; however, since the APC transgene was expressed from a

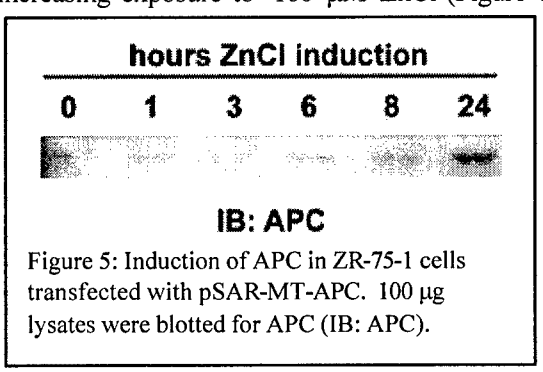

Figure 5: Induction of APC in ZR-75-1 cells transfected with pSAR-MT-APC. 100 μg lysates were blotted for APC (IB: APC).

different vector than the neomycin resistance gene, it is possible that the cells ceased expressing APC while still maintaining antibiotic resistance.

The difficulties that we have had with stable transfection of APC convinced us to alter our strategy for studying APC. Instead it appears that transient transfection (which does successfully increase the level of APC in most cell types) or study of endogenous protein alone is preferable. As mentioned above, however, the quality of most commercial antibodies to APC is very poor; this has been noted in the literature several times (for example, refs [14-16]). Publications have shown several commercially available antibodies—notably two sold by Santa Cruz and used by us for previous studies—to exhibit non-specific detection by western blot and immunostaining. Recent papers have even completely contradicted prior reports regarding APC localization, behavior in response to cell density, and nuclear-cytoplasmic shuttling.[14] Regarding our own work, we have also struggled with successfully detecting APC by western blot, however our difficulties have always been with lack of detection rather than detecting non-specific bands. Similarly, our immunofluorescence studies examined cytoplasmic APC rather than nuclear staining, so the published concerns about non-specific nuclear staining do not apply to our work. However, because of this lack of reliability in APC detection, we created two constructs expressing either the His_6 tag or a myc tag at the N-terminus of APC. These constructs were created by enzymatic digestion at the 5' end of the pcDNA3.1/Flag-APC construct (described above), followed by ligation of oligonucleotide "linkers" containing the DNA sequence for the appropriate tag. Oligonucleotides were synthesized by the Mayo Clinic Rochester DNA synthesis core facility. Antibodies to these epitope tags are well-characterized and should allow us to bypass the difficulties associated with many commercial APC antibodies. In our experience, the Flag tag antibodies do not excel in detecting large proteins that are expressed at low levels, such as APC. In addition to the myc- and His_6-tagged constructs, we have examined several new APC antibodies to find quality reagents for our studies, including two polyclonal antibodies, N-APC and M-APC[14] (kind gifts of I. Näthke at the University of Dundee), and a commercial monoclonal antibody, ALi 12-28 (AbCam). Our tests and reports in the literature indicate that all three of these antibodies are suitable for western blotting. ALi 12-28 can also be used for immunoprecipitation. M-APC was recently found to show non-specific nuclear staining,[14] and will therefore not be used for any immunostaining experiments that may be performed in the future.

To overcome the problems associated with APC antibodies, we have decided to explore additional techniques to complement our immunoprecipitation studies. We have developed reagents for two new assay systems, tandem affinity purification (TAP) and fluorescence resonance energy transfer (FRET). TAP involves the use of two distinct epitope tags that are fused to the protein of interest,[17] in this case APC. The tagged protein is then purified by binding the first epitope to beads, washing, eluting the bound complexes, binding the second epitope to additional beads, further washing, and then a final elution step. This two-fold process of binding a specific epitope and washing non-specific interactions off the beads should allow isolation only of molecules that form complexes with the tagged protein. We chose the Stratagene InterPlay TAP system, which uses a streptavidin-binding motif and a calmodulin-binding motif as the two epitope tags; both of these allow easy elution by biotin and EDTA, respectively, thus obviating the need for the protease cleavage step that is required in other TAP systems. The tagged APC construct (NTAP-APC) expresses successfully in BHK-21 and COS-1 cells at a size just slightly larger than the wildtype APC protein (Figure 6). We are currently working to determine what cell line(s) will be best for the TAP studies. The advantages of this system include the fact that, since only one protein of interest is tagged, we can use endogenous proteins known to interact with APC (e.g., β-catenin) as positive controls for the TAP assay. In addition, Stratagene provides a positive control to ensure that the system is working properly: vectors encoding either myocyte-enhancing factor 2A tagged with the streptavidin- and calmodulin-binding motifs, or myocyte-enhancing factor 2C tagged at the N-

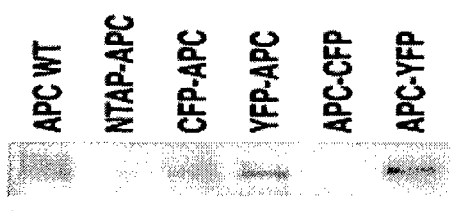

Figure 6: Expression of APC constructs in BHK-21 cells. Cells were transiently transfected and 100μg lysates were blotted for APC.

terminus with the Flag epitope. These vectors, when co-transfected into cells, will permit TAP extraction of the 2A/2C complex, which can then be detected using an antibody against the Flag tag.

The second assay we have begun exploring is FRET, which makes use of the unique excitation and emission spectra of fluorophores.[18] Emission of light from one fluorophore (called the "donor") can excite a second, nearby fluorophore (called the "acceptor") if their spectra are complementary, i.e., the emission spectrum of the donor overlaps the excitation spectrum of the acceptor. FRET experiments involve tagging two interacting molecules with a donor-acceptor pair; the donor is then excited by light of a wavelength that is within its excitation spectrum but outside of the excitation spectrum of the acceptor, causing the donor to fluoresce. When the tagged proteins are very close to one another (typically <70 Å apart), donor fluorescence can transfer enough energy to the acceptor to cause it to fluoresce as well. Reading the emission spectra of the two fluorophores, therefore, will indicate whether the tagged proteins are very close to one another (in which case the acceptor fluorescence is high and the donor fluorescence is lowered, due to quenching) or whether they are too far apart for FRET to occur (in which case only the donor fluorescence is high). These studies will be done in collaboration with the laboratory of Dr. Laurence J. Miller at Mayo Clinic Scottsdale. This group has extensive experience with fluorescence studies and FRET: they have published papers using these techniques,[19, 20] and have an advanced fluorescence microscopy setup that permits monitoring of both fixed and live cells. Our strategy for using FRET to study MUC1 and APC is initially to fix and mount transfected cells (constructs described below) on slides using 2% paraformaldehyde and Vectashield mounting medium (Vector Labs); this protocol retains fluorescent protein activity and protects the fluorescence from bleaching. The cells will be examined for membrane localization of MUC1 and cytoplasmic localization of APC. Cells meeting these criteria will be used for FRET analysis; the QED In Vivo (Media Cybernetics) software permits analysis of select regions within cells—in this case, only the perimembrane area—to eliminate non-specific FRET signals from improperly localized proteins. This precludes one of the major concerns with FRET studies, namely non-specific fluorescence from proteins that have not yet exited the biosynthetic cascade and are therefore capable of showing energy transfer that is not relevant to the interactions of the proteins being analyzed.

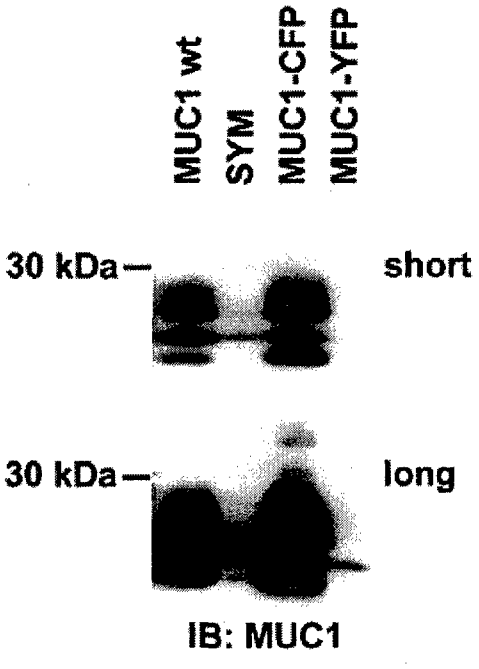

Figure 7: Expression of MUC1 fluorescent fusion constructs. Two exposures, short and long, were taken of a blot for MUC1 (IB: MUC1).

For these studies, we have chosen to fuse MUC1 and APC to either cyan fluorescent protein (CFP) as the donor or yellow fluorescent protein (YFP) as the acceptor. This strategy has been employed before to study MUC1 interactions with ICAM-1 in the laboratory of Dr. Judith C. Hugh (University of Edmonton, Canada), who has kindly given us three MUC1 constructs for FRET experiments. These constructs are: YFP fused to the N-terminal extracellular domain of MUC1 (called SYM for "signal sequence-YFP-MUC1"),[2] YFP fused C-terminal to the MUC1-CT (called MUC1-YFP), and CFP fused C-terminal to the MUC1-CT (called MUC1-CFP). These proteins can be seen in Figure 7. Note that the SYM construct should and does appear at the same size as wildtype MUC1, as the antibody used detects the MUC1-CT, not the extracellular portion where YFP has been added in this construct. However, the MUC1-CFP and MUC1-YFP constructs should show an increase in size, corresponding to the additional 25 kDa added by the fluorescent proteins. Though a significant amount of MUC1-CT appears at the wildtype size (likely due to expression of MUC1 without the fluorescent fusion protein), with longer exposure several bands are visible at sizes ranging from 30-40 kDa, indicating that some cells do express the complete fusion proteins. The presence of several bands is due to the nature of MUC1, which shows a

widely variable pattern of glycosylation and/or phosphorylation, resulting in numerous bands in western blots. It is actually encouraging that we see multiple larger bands in the MUC1-CFP and MUC1-YFP constructs, as this most likely indicates that these constructs are being glycosylated and therefore that the fluorescent tags do not interfere with normal cellular processing of MUC1. In addition, any non-fluorescent protein expressed in the MUC1-CFP or MUC1-YFP transfected cells should not interfere with FRET studies, as only cells expressing the fluorophore will be examined. In addition to the MUC1 fluorescent constructs, we have created similar expression vectors for APC where the fluorophore is fused either N-terminal (YFP-APC and CFP-APC) or C-terminal (APC-YFP and APC-CFP) to the APC cDNA. For the N-terminal fluor constructs, the APC cDNA was simply excised from pcDNA3.1/Flag-APC using a restriction enzyme digest, and then ligated into the pECFP-C1 or pEYFP-C1 vectors. These two vectors were obtained from our collaborator, Dr. Gunnar Hansson, and have been modified (three point mutations) by his group to prevent dimerization of the CFP and YFP proteins. For the C-terminal fluor constructs, CFP and YFP were amplified by PCR using the pECFP-N1 or pEYFP-N1 vectors (gifts of L. Miller) as templates, and ligated into the pcDNA3.1/Flag-APC vector. All four fluorescent APC proteins express at a slightly larger size than wildtype APC (Figure 6), as is expected. Both the MUC1 and APC fluorescent fusion constructs express in several cell lines (COS-1, COS-7, BHK-21, and CHO-K1 have been tested so far) and do fluoresce, indicating that the fusion proteins are successfully expressed and folded (Figure 8). We have finished testing the MUC1 and APC fluorescent constructs, and are currently optimizing transfection conditions prior to beginning FRET studies. FRET will be used to complement many of the experiments we have already proposed, but intended to analyze by immunoprecipitation, such as assessing the effect of EGF stimulation or ErbB kinase inhibition on the MUC1-APC interaction. In addition to serving as confirmation for the results of immunoprecipitation assays, FRET also offers the advantage of being able to visualize live cells, and therefore to see physiological responses to stimuli and measure the kinetics of alterations in protein-protein interactions.

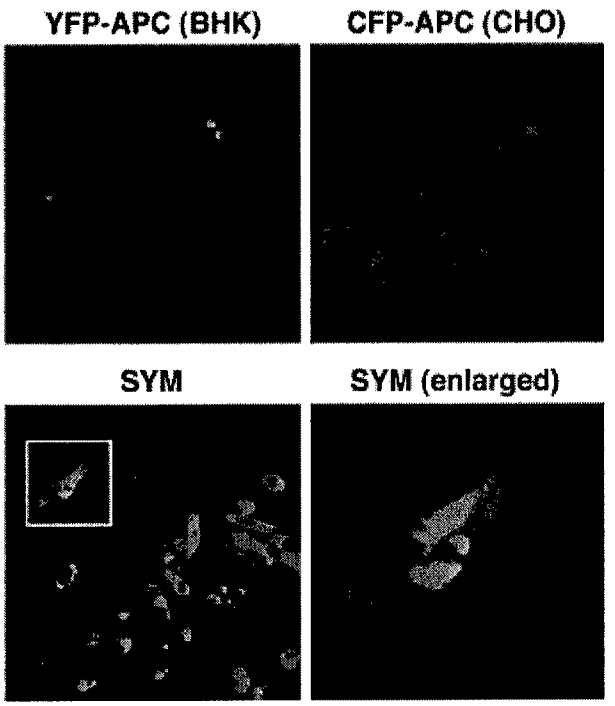

Figure 8: Confocal images of APC and MUC1 fluorescent fusion proteins. Lower right panel is enlarged image of boxed area in lower left panel.

Finally, recent experiments have given some exciting results regarding a possible role for MUC1 in β-catenin signaling. MDA-MB-468 cells were transfected with small interfering RNA (siRNA) oligonucleotides directed against the MUC1 mRNA (MUC1 SmartPool from Dharmacon). SiRNA technology is a powerful tool that takes advantage of cells' inherent defense systems against double stranded RNA.[21] The basic strategy is to transfect cells with short oligonucleotides of complementary sequence to the mRNA of the gene of interest. The siRNA oligonucleotides will hybridize to the mRNA, creating a short stretch of double stranded RNA, which induces the cell to destroy that mRNA without translating it into protein. The overall effect of siRNA transfection is to create a short-term (usually 24-96 hours) knockdown of the molecule of interest, in this case MUC1. MDA-MB-468 cells were transfected with Lipofectamine2000 (Invitrogen) and either 10, 50, or 100 nM SmartPool oligonucleotides directed against MUC1, or 50 nM oligonucleotides directed against lamin as a control ("mock"). Cells were lysed 48 hours after transfection and 50 μg lysates were analyzed by western blot. SmartPool treatment successfully decreased the amount of MUC1 in the cells (Figure 9). The antibody

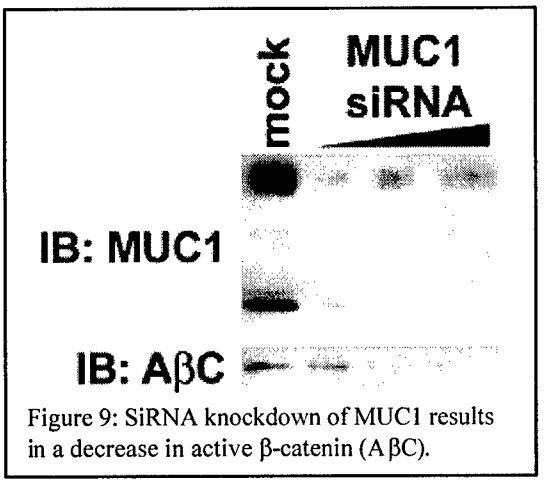

Figure 9: SiRNA knockdown of MUC1 results in a decrease in active β-catenin (AβC).

used to blot for MUC1 detects the extracellular tandem repeat domain, hence the wide range of bands corresponding to differentially glycosylated isoforms of MUC1. Interestingly, the knockdown of MUC1 correlated with a decrease in the level of active β-catenin as measured by an antibody that specifically recognizes β-catenin that is not phosphorylated on the N-terminal regulatory residues Ser37 and Thr41.[22] It is important to note that we have never detected co-immunoprecipitation between MUC1 and APC in this cell line; this could reflect a failure of the proteins to associate in this cell line, or simply an inability to detect a low-level interaction using the antibodies available. Regardless, the correlation of MUC1 levels with β-catenin activity fits well with our hypothesis that the MUC1-APC association may be part of a larger, protective role MUC1 has regarding β-catenin signaling. Future plans to further analyze these results include determining at what level MUC1 affects β-catenin activity: northern blots or RT-PCR for β-catenin mRNA and western blots for total β-catenin levels will show if MUC1 affects transcription or translation of β-catenin. Similarly, pulse-chase analysis can be used to determine if the presence of MUC1 affects β-catenin protein stability. To analyze the effect of MUC1 on the β-catenin destruction complex, we currently have lithium chloride (an inhibitor of GSK3β) and constructs expressing wildtype and dominant negative GSK3β, in addition to our reagents for the study of APC. We will confirm that the level of unphosphorylated β-catenin correlates with β-catenin activity; this can be done through western blots for β-catenin targets such as cyclin D1 and c-myc, and through luciferase assays using the TOPFLASH and FOPFLASH constructs (luciferase driven by consensus and scrambled TCF/β-catenin binding sites, respectively). It will also be important to analyze other breast cancer cell lines to determine whether the correlation of MUC1 levels with active β-catenin is a general phenomenon. We believe that these studies will nicely complement our work regarding the MUC1-APC interaction, and will provide evidence for a functional role for MUC1 in regulating β-catenin activity.

In summary, we have shown that APC interacts preferentially with wildtype MUC1 and not the Y0 mutant which lacks the seven tyrosine residues of the cytoplasmic tail. We have overcome APC antibody-associated difficulties by testing new antibodies and creating tagged constructs and inducible cell lines for APC expression. We have developed reagents and protocols for two new assays, tandem affinity purification (TAP) and fluorescence resonance energy transfer (FRET). Finally, we have determined that siRNA-mediated knockdown of MUC1 in MDA-MB-468 breast cancer cells correlates with a reduction in active β-catenin, indicating that MUC1 may have a protective role regarding the ability of β-catenin to signal in breast cancer. Our future studies will use these new techniques to explore the role of MUC1 and APC in mediating β-catenin and ErbB signaling in breast cancer.

Key Research Accomplishments

- APC appears to preferentially associate with wildtype MUC1 as compared to MUC1 lacking the 7 tyrosines in the cytoplasmic tail, indicating that MUC1 phosphorylation might be important in this interaction.

- ZR-75-1 and MDA-MB-468 cells were stably transfected with zinc-inducible constructs expressing APC or a lacZ control (pSAR-MT-APC and pSAR-MT-lacZ). APC expression could be increased in the ZR-75-1 line by treatment with 100 μM ZnCl.

- Zinc-inducible APC expression was tested and found to be too inconsistent for immunoprecipitation studies, despite several controls and numerous attempts to optimize the protocol and techniques.

- The problem of unreliable antibodies for APC was overcome by two means: (1) development of vectors expressing myc-tagged or His6-tagged full-length APC and (2) testing of several new antibodies based on recommendations from an experienced researcher in the field of APC studies (I. Näthke).

- A construct for tandem affinity purification (TAP) has been created, fusing streptavidin- and calmodulin-binding motifs 5' to the APC cDNA. This construct has been tested by expression in cells and is currently being used for TAP experiments.

- I have successfully learned and performed the TAP assay (InterPlay from Stratagene), which was never before used in our laboratory.

- Four constructs expressing APC tagged with a fluorescent protein (either CFP or YFP) have been developed and tested in several cell lines. All four constructs express successfully and can be used for a variety of assays, including FRET.

- I am currently learning the FRET assay, with the generous assistance of Dr. Kaleeckal Harikumar, a research associate in Dr. Laurence Miller's laboratory. Our initial work has been with fixed and mounted cells; however, we anticipate using live-cell FRET techniques in the future. Our laboratory has used confocal and fluorescence microscopy extensively in the past and currently, but this is the first time that FRET has been used for our studies of MUC1.

- MUC1 has been successfully and reproducibly knocked down in a breast cancer cell line, MDA-MB-468, using siRNA technology. As these cells are notoriously difficult to transfect, it is amazing what that such a dramatic level of MUC1 knockdown was attained with Lipofectamine2000 and the SmartPool oligonucleotides.

- The level of MUC1 knockdown in MDA-MB-468 cells via siRNA correlates with the amount of active β-catenin as measured by an antibody that specifically recognizes β-catenin that is not phosphorylated at Ser37 or Thr41.

Reportable Outcomes

- Hattrup, C.L., Fernandez-Rodriguez, J., Schroeder, J.A., Hansson, G.C., and Gendler, S.J. "MUC1 can interact with adenomatous polyposis coli in breast cancer" *Biochem Biophys Res Comm* 2004 Apr 2;316(2):364-9. Note—the work represented in this publication was largely complete prior to the beginning of the grant period and is therefore not appended to this report.

- Conference attendance at Keystone Symposia on Cancer and Development / The Role of Microenvironment in Tumor Induction and Progression (joint conferences), February, 2005. A poster was presented, entitled, "Characterization of the MUC1-APC interaction in breast cancer."

- Quarterly presentation of publications from high-impact journals as part of the "Current Events in Tumor Biology" journal club that is videoconferenced between the Mayo Clinic Rochester, Scottsdale, and Jacksonville sites. This journal club is attended by principal investigators, postdoctoral fellows, and graduate students in the Tumor Biology training program and takes place every week.

- Frequent presentation (every 6-8 weeks) of publications as part of a laboratory group journal club. The journal club occurs every other week and is attended by members of Dr. Gendler's laboratory and that of Dr. Pinku Mukherjee, at Mayo Clinic Scottsdale.

- Presentation (every 2-3 months) of my own work in lab meeting. Lab meeting occurs every other week and is attended by Dr. Gendler's and Dr. Mukherjee's groups.

- Yearly presentation of my own work as part of "Research Project Update," as required for all postdoctoral fellows and graduate students in research at Mayo Clinic Scottsdale. Research Project Update is attended by all employees involved in research in Scottsdale, including animal care technicians, laboratory technicians, postdoctoral fellows, graduate students, and principal investigators.

- Yearly presentation of my own work as a requirement of the Tumor Biology training program. These presentations are videoconferenced between all three Mayo Clinic sites and are attended by graduate students, postdoctoral fellows, and principal investigators.

- Development of breast cancer lines (ZR-75-1 and MDA-MB-468) expressing APC or lacZ under a zinc-inducible (metallothionein) promoter, the pSAR-MT-APC and pSAR-MT-lacZ constructs.

- Development of constructs for analysis of APC:
  1. fused to cyan or yellow fluorescent proteins for FRET
  2. tagged with streptavidin-binding and calmodulin-binding motifs for TAP
  3. tagged with His_6 or myc for mammalian expression / purification.

Conclusions

This project was designed to study the interaction of MUC1 with the APC tumor suppressor. Our work prior to the grant period showed that MUC1 and APC interact in a variety of systems, including lactating mouse mammary gland and in human breast tumors. Interestingly, the association could be increased by treatment of mammary glands or a breast cancer cell line with exogenous EGF, indicating that the ErbB pathway may be important for regulating MUC1-APC association. Yet more important was the finding that the level of MUC1-APC interaction was increased in primary human breast tumors with lymph node metastases, indicating that these proteins may somehow facilitate metastasis. This project, therefore, set out to define the physiological conditions under which MUC1 and APC interact, and to determine the biological significance of the association in relation to β-catenin and/or ErbB signaling.

Our work during Year 1 of this grant has focused on determining the biological significance of the MUC1-APC interaction. Given the published literature regarding the poor quality of many APC antibodies, our primary concern was to set up reliable, consistent assays with which to perform our studies. To this end we have established two experimental systems never previously used in our laboratory: tandem affinity purification (TAP) and fluorescence resonance energy transfer (FRET). These assays are extremely powerful tools that will complement and expand our previously proposed studies. For use in FRET, we have created fluorescently-tagged APC constructs which express full-length APC as a fusion protein to either cyan fluorescent protein or yellow fluorescent protein. For the TAP system we created a construct expressing APC tagged at the 5' end with streptavidin- and calmodulin-binding motifs. In addition to these, we have created vectors expressing APC with the His_6 or myc tags, both of which have well-characterized antibodies. These constructs will allow us to avoid some of the problems associated with poor APC antibody quality, and will provide confirmation for the results derived using APC antibodies. We have also expressed APC in breast cancer cell lines under a zinc-inducible promoter (pSAR-MT-APC); though these cells proved too inconsistent for immunoprecipitation studies, the ability to induce APC expression *in vitro* could be quite useful for later studies.

We have determined that APC preferentially interacts with wildtype MUC1 as compared to MUC1 lacking the seven tyrosine residues of the cytoplasmic tail (MUC1-Y0). This result quite likely indicates a need for phosphorylation of the MUC1-CT to stimulate its association with APC, and ties in well with our previous findings that EGF stimulates the MUC1-APC interaction. EGFR has been shown to phosphorylate MUC1 on tyrosine, thus it is probable that EGF treatment of cells or mouse mammary gland activates EGFR, which then phosphorylates MUC1 on the cytoplasmic tail (see Figure 1 for sites) to stimulate its association with APC. We have additional cell lines bearing single or double tyrosine mutations to delineate exactly what residues are involved in the MUC1-APC interaction.

Interestingly, we have correlated the siRNA-mediated knockdown of MUC1 in MDA-MB-468 cells with a reduction in active β-catenin levels. Though there is much work to be done to confirm and explain these results, this finding ties in well with the concept of MUC1 serving a protective role regarding β-catenin signaling. It is probable, given the overexpression of both MUC1 and β-catenin in many breast cancers, that they somehow cooperate in tumor progression. This is supported by the finding that the level of MUC1-β-catenin interaction increases in breast tumor metastases as compared to primary tumors and that the association is largely absent from surrounding normal tissues. Given that APC is known to decrease β-catenin activity, its interaction with MUC1 most likely represents a mechanism of disrupting APC-mediated downregulation of β-catenin. This too ties in with our finding that MUC1 binds APC most readily in breast tumors that have metastasized to lymph nodes. Our current model for understanding the MUC1-APC interaction envisions MUC1 cooperating with β-catenin in oncogenic signaling: this may occur by MUC1 stabilizing active β-catenin (supported by our siRNA data), or by MUC1 interacting with members of the β-catenin degradation complex, such as APC, in order to disrupt its function. This activity could be stimulated by oncogenic signals such as active Src or EGFR, both of which increase MUC1 binding to β-catenin, and which may influence the MUC1-APC association as well. Our upcoming studies will be aimed towards delineating the role of MUC1 and APC in β-catenin signaling in breast cancer cells, as this area of study is most likely to reveal the importance of the MUC1-APC interaction in breast cancer and metastasis.

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